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Composition of the Essential Oil of Alfalfa

Takayasu Kami

The essential oil of alfalfa was isolated by steam distillation of the fresh grass with a yield of 0.009%. The essential oil was analyzed by combined gas chromatography–mass spectrometry and gas chromatographic comparison with authentic specimens, and 67 compounds consisting of hydrocarbons, aldehydes, ketones, alcohols, esters, acids, phenol, lactones, sulfur compounds, and miscellaneous were identified. Quantitative analysis was further carried out on the essential oil, and alfalfa oil was relatively rich in esters and alcohols.

In course of a series of studies on the aromatic constituents of forage crops, the present work analyzed the essential oil of alfalfa by means of combined gas chromatography–mass spectrometry and gas chromatography, following that of the previous papers (Kami, 1975, 1977, 1978, 1981) on the analyses of the essential oils of Hybridsorgo, Sudangrass, red and Ladino white clovers, and soiling dent corn. Some studies on the volatile components of alfalfa flowers (Loper et al., 1971) and of alfalfa leaves and stems (Buttery and Kamm, 1980) had been carried out so far.

Since alfalfa (*Medicago*) occupies an important role for the nutrition of domestic animals as the leguminous pasture plants, the different species of alfalfa are widely cultivated over the world. In the southwestern warm district of Japan, however, alfalfa is not cultivated so much because of inadaptation to the climate and soil of this district situated in the monsoon zone. Therefore, alfalfa is imported into Japan in the form of alfalfa pellets or alfalfa hay cubes.

EXPERIMENTAL SECTION

Materials. Alfalfa (Du Puits, *Medicago sativa* L.) was cultivated on a farm of Hiroshima Agricultural College,

and the aerial parts were harvested in June 1980 by sickle. The harvest time corresponded to the flowering stage of alfalfa.

Isolation of the Essential Oil. The fresh aerial parts with flowers (230 kg), after being cut by a cutter, were steam distilled in 25-kg lots for 1 h under 0.7 kg/cm^2 distillation kettle pressure by using a boiler in a cannery of the Faculty of Applied Biological Science, Hiroshima University, and yielded about 180 L of cloudy distillate in a water-cooled trap and 28.4 g of colorless aqueous condensate in an ice–water-cooled trap. After the cloudy distillate of the water-cooled trap was saturated with sodium chloride, 2-L lots were extracted twice with 300 mL of distilled diethyl ether in the same way as described in a previous paper (Kami, 1975) to yield an yellow-brown oil with a grassy-smelling odor (21.8 g). The essential oil was stored in a sealed glass tube at 3 °C, as was the aqueous condensate from the ice–water-cooled trap.

Fractionation of the Essential Oil. A portion of the essential oil was sequentially shaken with 5% sulfuric acid, 5% sodium carbonate, and 5% sodium hydroxide aqueous solutions, 3 times with each 20 mL. The pH of each aqueous solution was reversed, and each solution was reextracted with diethyl ether to obtain basic (55 mg, 3.2%), acidic (343 mg, 20.0%), and phenolic (37 mg, 2.2%) fractions, respectively. The remaining neutral oil layer was successively extracted in *n*-pentane, diethyl ether, and diethyl ether–methanol (90:10) with silicic acid to separate

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it into nonpolar (354 mg, 20.7%), polar I (766 mg, 44.8%), and polar II (156 mg, 9.1%) fractions, respectively (Kami, 1977). Among them, the acidic fraction was further converted into the methyl esters with diazomethane (Vorbeck et al., 1961).

Analysis of the Essential Oil. In the beginning, the unfractionated essential oil was analyzed by combined gas chromatography–mass spectrometry (GC–MS). A Hitachi Model RMU-6MG mass spectrometer was used. The GC column was a 0.28 mm i.d. × 50 m glass capillary column coated with PEG 20M, and the column temperature was programmed from 60 to 200 °C at 2 °C/min. The carrier gas was helium. The mass spectrometer was worked at 70-eV ionizing voltage, 3200-V ion accelerating voltage, and 200 °C ion source temperature. In the next phase, the fractions of the essential oil, except for the basic fraction, and the unfractionated essential oil were analyzed by programmed temperature gas chromatography (GC). An FID-type Shimadzu GC-7APF gas chromatograph was used. The GC column was a U-shaped 3 mm i.d. × 3 m glass column packed with 5% Thermon 3000 on 80–100-mesh Chromosorb W, and the column temperature was maintained at 40 °C for the first 5 min and then increased at a rate of 4 °C/min to 250 °C (nitrogen flow rate, 40 mL/min; injection port temperature, 300 °C). On the other hand, for separation of the overlapped compounds in GC of Thermon 3000 column, a U-shaped 3 mm i.d. × 3 m glass column packed with 5% silicon OV-101 on 80–100-mesh Chromosorb W was used to the same gas chromatograph. The column temperature was maintained at 40 °C for the first 5 min and then increased at a rate of 4 °C/min to 270 °C (nitrogen flow rate, 30 mL/min; injection port temperature, 300 °C). A portion of 5% sulfuric acid soluble fraction was heated with 3 mL of 8% sodium hydroxide aqueous solution, and the regenerated gases of amines were analyzed by isothermal GC with a Thermon 1000 (15%) plus PEG 1000 (15%) plus KOH (10%)–Shimalite column at 70 °C (Kami, 1975). For the analysis of low-boiling compounds, the headspace vapors of the unfractionated essential oil and ice–water-cooled trap were directly chromatographed by using a Carbowax 1500 (10%)–Diasolid L column at 45 °C (Kami et al., 1972).

Percentage Composition of the Essential Oil. The relative peak areas in GC of the unfractionated essential oil were calculated from the tracing paper cutout weights of the peaks in the chromatogram of the Thermon 3000 column by using a Mettler electronic balance. The relative peak areas of the unfractionated essential oil were then multiplied by 0.968 (derived from the residual 96.8%; 3.2% of the yield is the basic fraction, because amines could not be detected by GC of the unfractionated essential oil). For separation of the overlapped compounds in chromatogram of Thermon 3000 column, the unfractionated essential oil was next chromatographed by a silicone OV-101 column. The ratios of the separated compounds by this column were calculated from the tracing paper cutout weights of the peaks and multiplied by the relative peak areas of the Thermon 3000 column.

RESULTS AND DISCUSSION

Identification of the Components in the Essential Oil. The fresh crop of alfalfa was distilled with steam and then extracted with diethyl ether to collect the essential oil. The essential oil was first analyzed by GC–MS of the PEG 20M column without any fractionation. Among 114 peaks in all, 67 compounds consisting of 2 hydrocarbons, 11 aldehydes, 2 ketones, 7 alcohols, 32 esters (21 ethyl esters, 7 acetates, 2 diethyl esters, 1 methyl ester, and benzyl benzoate), 5 acids, phenol, 3 lactones, 2 sulfur

compounds, and 2 miscellaneous, which are indicated in Table I, were identified through comparison of mass spectra with those of authentic specimens and/or with authentic spectra (Stenhagen et al., 1969). The other peaks could not be identified because their mass spectra were those of mixed state or indistinct state.

On the basis of the GC–MS results, the acidic, phenolic, nonpolar, and polar fractions and the unfractionated essential oil were next analyzed by programmed temperature GC of Thermon 3000 column. These peak assignments were based on the coincidence of the peaks when the authentic specimens were added to each sample. After the 5% sulfuric acid soluble fraction was heated with alkali, the headspace vapor was analyzed by isothermal GC, and also, the headspace vapors of the unfractionated essential oil and ice–water-cooled trap were directly analyzed by isothermal GC. These peak assignments were based on the coincidence of t_R with those of the authentic specimens. In the GC analysis of the nonpolar fraction, the normal alkanes from C_{25} to C_{31} were detected. The normal alkanes less than C_{24} could not be detected because of the small and many peaks. In the normal alkanes above C_{25}, odd-numbered carbons predominated over even-numbered carbons, and *n*-pentacosane was the major constituent of the nonpolar fraction. The acidic fraction was chromatographed directly or via methylation, and the normal carboxylic acids from acetic acid to stearic acid and isobutyric, 2-methylbutyric, isovaleric, linolic, benzoic, and phenylacetic acids were detected. Benzoic acid was the major constituent of the acidic fraction. From the phenolic fraction, phenol and *p*-cresol were detected, and from the basic fraction, *sec*-amylamine and tri-*n*-propylamine were detected. The GC pattern of the polar I fraction resembled to that of the unfractionated essential oil, but the decrease of the peaks corresponding to low-boiling compounds, acids, and dimethyl sulfone was observed. 6,10,14-Trimethylpentadecan-2-one was the major constituent of the polar I fraction. The GC pattern of the polar II fraction was the small peaks on the whole. From the GC of the headspace vapors of the unfractionated essential oil and ice–water-cooled trap, acetaldehyde, methyl formate, ethyl formate, acetone, ethyl acetate, isovaleraldehyde, ethyl propionate, and ethanol were detected.

From the above results, it became clear that many esters and acids are contained in the essential oil of alfalfa, while hydrocarbons, ketones, and phenols are rather few. Such tendency is also found in clovers (*Trifolium*). However, lactones and sulfur compounds are not included in clovers, and furthermore, ethyl levulinate, ethyl 3-hydroxy-hexanoate, 4-hexanolide, 2,3-dimethyl-2-nonen-4-olide, dimethyl disulfide, and phenylacetonitrile are not included in the essential oils of sorghums, clovers, and soiling dent corn (Kami, 1975, 1977, 1978, 1981). On the other hand, 1-octen-3-ol and many terpenoids, which Buttery and Kamm (1980) identified as the characteristic components in the volatile oil of alfalfa leaves and stems, could not be detected in spite of the careful examination of mass spectra patterns. Futher investigation is necessary to determine whether these compounds depend upon the discrepancy of alfalfa species or not.

Percentage Composition of the Essential Oil. The percentage composition of the essential oil of alfalfa was subsequently calculated to clarify the aroma character. The result is listed in Table I. The compounds containing more than 3% of essential oil were ethanol, ethyl acetate, acetic acid, dimethyl sulfone, benzaldehyde, acetone, and ethyl palmitate, in the order of abundant percentage. In the total amounts by functional groups, hydrocarbons,

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Table I. Compounds Identified from the Essential Oil of Alfalfa and Their Percentage Compositions

compound	peak no.[a]	%[b]	compound	peak no.[a]	%[b]
hydrocarbons (2)			acids (5)		
p-cymene	31	0.3	acetic acid	43	8
n-pentadecane	48	<0.1	isovaleric acid	58[c]	0.2
aldehydes (11)			2-methylbutyric acid	58[c]	2
acetaldehyde	1	0.2	caproic acid	69	0.4
3-methylbutanal	6[c]	0.6	benzoic acid	96	0.5
1-pentanal	10	0.5	esters (32)		
1-hexanal	16[c]	0.5	ethyl formate	3[c]	0.3
1-heptanal	23	0.1	ethyl acetate	5	9
1-octanal	33	<0.1	ethyl n-butyrate	13	0.6
1-nonanal	42	0.5	ethyl 2-methylbutyrate	14	2
furfural	45	2	ethyl isovalerate	15	2
1-decanal	49	0.1	isoamyl acetate	19	0.2
benzaldehyde	50	4	ethyl n-valerate	20	0.2
phenylacetaldehyde	57	1	ethyl caproate	28	1
ketones (2)			n-hexyl acetate	32	0.4
acetone	3[c]	4	trans-3-hexenyl acetate	35	0.7
6,10,14-trimethylpentadecan-2-one	82	2	cis-3-hexenyl acetate	36	2
alcohols (7)			ethyl oenanthate	37	0.3
ethanol	6[c]	12	1-octen-3-yl acetate	41	0.3
1-hexanol	38	0.6	ethyl caprylate	44	2
trans-3-hexen-1-ol	39	0.7	ethyl pelargonate	51	0.5
cis-3-hexen-1-ol	40	2	diethyl malonate	53	0.2
linalool oxide	46	0.7	ethyl levulinate	55	0.3
benzyl alcohol	70[c]	2	methyl benzoate	56	0.3
2-phenylethanol	72	0.4	ethyl benzoate	59[c]⎫	1
phenols (1)			diethyl succinate	59[c]⎭	
phenol	76	0.6	ethyl 3-hydroxyhexanoate	59[c]	0.2
lactones (3)			benzyl acetate	63	2
4-hexanolide	61	0.5	ethyl phenylacetate	66	1
2,3-dimethyl-2-nonen-4-olide	84	0.2	2-phenylethyl acetate	68	0.3
dihydroactinidiolide	92	0.7	ethyl laurate	70[c]	0.2
sulfur compounds (2)			ethyl myristate	80	0.6
dimethyl disulfide	16[c]	0.3	ethyl pentadecanoate	83	0.9
dimethyl sulfone	71	4	ethyl palmitate	89	3
miscellaneous (2)			ethyl heptadecanoate	93	0.1
phenylacetonitrile	73	0.6	ethyl stearate	97	0.6
methyl eugenol	77	0.9	ethyl oleate	98	0.4
			benzyl benzoate	101	0.1

[a] Peak numbers were given in the order of appearance in the programmed temperature GC (Thermon 3000 column) of the unfractionated essential oil. [b] Percentages were calculated from 0.968 (the peak area percents of the unfractionated essential oil). [c] The percentages of the overlapped compounds of same peak numbers were calculated from the ratios of the peak areas on the programmed temperature GC (silicone OV-101 column) of the unfractionated essential oil.

aldehydes, ketones, alcohols, esters, acids, phenols, lactones, sulfur compounds, and miscellaneous were ca <1, 10, 6, 18, 33, 11, <1, 1, 4, and 2%, respectively, in the essential oil. In comparison with the previous results (Kami, 1978, 1981), the essential oil of alfalfa was relatively rich in esters and alcohols, while hydrocarbons and phenols were less abundant. It was found out that such tendency is similar to that of clovers, especially to Ladino white clover. Accordingly, from the qualitative and quantitative analyses of the aromatic constituents of alfalfa, it may be said that alfalfa is as excellent as clovers in its palatability for domestic animals. However, further investigation of the relation between the aroma of alfalfa and its palatability for domestic animals is necessary.

CONCLUSION

From the essential oil of alfalfa, 67 compounds including 2 hydrocarbons, 11 aldehydes, 2 ketones, 7 alcohols, 32 esters, 5 acids, phenol, 3 lactones, 2 sulfur compounds, and 2 miscellaneous were identified by GC–MS and GC. In addition, 30 compounds including 7 normal alkanes (C_{25}–C_{31}), methyl formate, ethyl propionate, 15 normal carboxylic acids (C_3–C_{18}, except for C_6), isobutyric acid, linolic acid, phenylacetic acid, p-cresol, sec-amylamine, and tri-n-propylamine were detected by GC alone. Many esters and acids were included in the essential oil. In the quantitative analysis by functional groups, the essential oil was relatively rich in esters and alcohols. Such results

were similar to the those of clovers.

ethyl levulinate, 539-88-8; methyl benzoate, 93-58-3; ethyl benzoate, 93-89-0; diethyl succinate, 123-25-1; ethyl 3-hydroxyhexanoate, 2305-25-1; benzyl acetate, 140-11-4; ethyl phenylacetate, 101-97-3; 2-phenylethyl acetate, 103-45-7; ethyl laurate, 106-33-2; ethyl myristate, 124-06-1; ethyl pentadecanoate, 41114-00-5; ethyl palmitate, 628-97-7; ethyl heptadecanoate, 14010-23-2; ethyl stearate, 111-61-5; ethyl oleate, 111-62-6; benzyl benzoate, 120-51-4.

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# Occurrence of an Unusual Leucoanthocyanidin and Absence of Proanthocyanidins in Sorghum Leaves

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Condensed tannins of sorghum seeds could, if also present in vegetative tissue, lower the digestibility of the forage. In order to eliminate interference by pigments such as chlorophyll and anthocyanins in tannin assays of forage, we adsorb tannin and related flavanols on insoluble poly(vinylpyrrolidone) (PVP). After washing out interfering materials we heat the PVP in HCl/butanol to convert the bound tannins to anthocyanidins which are measured spectrophotometrically. By use of this assay, no tannins were found in leaf tissue of the 47 sorghum lines examined. However, leaf tissue from 16 of the lines was found to contain a PVP-binding material with properties corresponding to a monomeric flavan-4-ol (designated a leucoanthocyanidin). The leucoanthocyanidin, which is also present in seed of these varieties, has been tentatively identified as apiforol (4',5,7-trihydroxyflavan-4-ol). This unusual monomeric flavan-4-ol, which has not been previously reported from plants, yields the yellow anthocyanidin, apigeninidin, when heated in aqueous acid and at low temperatures in acid/alcohol mixtures is converted to an unidentified unstable pink anthocyanidin.

Tannins are the most important group of secondary metabolites involved in plant defense (Swain, 1979). Tannins present in the grain of certain sorghum varieties confer agronomic benefits such as resistance to bird depredation (McMillian et al., 1972) and to preharvest seed molding (Harris and Burns, 1973). Tannins bind certain proteins very strongly (Hagerman and Butler, 1981) and thus diminish the digestibility and nutritional value of high tannin sorghum grain (Price et al., 1979). Sorghum seed tannins are of the "condensed" type (Strumeyer and Malin, 1975), oligomers of flavan-3-ols. Because of their propensity for depolymerization in acid solution to yield anthocyanidin pigments, condensed tannins are classified as proanthocyanidins; those from sorghum form cyanidin and are therefore called procyanidins (Gupta and Haslam, 1978). Certain monomeric flavanols such as flavan-3,4-diols and flavan-4-ols can also give rise to anthocyanidins (Scheme I) and are therefore distinguished from the oligomeric flavan-3-ols by the name "leucoanthocyanidin" (Weinges et al., 1969).

Relatively little is known about the occurrence and distribution of proanthocyanidins and leucoanthocyanidins in sorghum tissues other than grain. As a result of injury or physiological stress, sorghum leaf tissue frequently develops red coloration due to anthocyanidins and/or anthocyanins (Bate-Smith, 1969). Leucoanthocyanidins

Scheme I
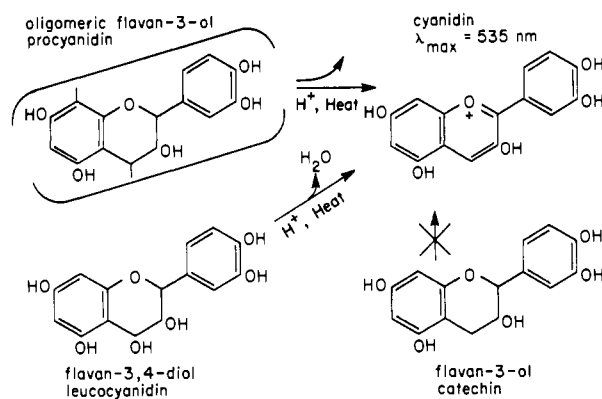
could be the precursors of these pigments. If proanthocyanidins serve as a deterrent to herbivores and pathogens (Swain, 1979), they might be expected to occur in the vegetative tissue of the plant, as well as the seed. Proanthocyanidins present in forage could diminish its nutritional value. Jung and Fahey (1981) reported that removal of unidentified phenolic materials from alfalfa increases its digestibility.

We have adapted an anthocyanidin formation assay to vegetative tissue, eliminating interference by chlorophyll and endogenous anthocyanin or anthocyanidin pigments. We found a leucoanthocyanidin in the leaf tissue of 12 of the 43 sorghum lines we examined but no proanthocyanidins in the leaf tissue of any of the lines.

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